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High Differentiation among Populations of Green Foxtail, *Setaria viridis*, in Taiwan and Adjacent Islands Revealed by Microsatellite Markers

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Abstract: *Setaria viridis* (L.) Beauv., or green foxtail, is native to Eurasia and is the putative ancestor of foxtail millet. Due to the advantageous genetic characteristics of *S. viridis*, it is a model species for C4 plants. However, *S. viridis* has seriously spread to the agricultural system around the world because of its wide adaptability. This study is aimed to understand the distribution of *S. viridis* in Taiwan, and also to investigate the genetic diversity and relationships among different wild populations. A total of 141 *S. viridis* collected at 10 sites with sampling sizes ranging from 8 to 24 plants in Taiwan were analyzed by 13 highly polymorphic SSR markers, and 6.1 alleles per locus were detected in our study. The relationships of collected *S. viridis* mostly corresponded to its distribution in different parts of Taiwan revealed by PCoA and phylogenetic tree. Similarly, the results for population structure showed the significance of collecting site or geographical factors. Finally, the extent of gene flow was studied with the genetic differentiation (F_{ST}) and Nm values, and two *S. viridis* populations were found to significantly contain the existence of gene-flow events. In conclusion, *S. viridis* showed a pattern of low diversity and heterozygosity within a population, but high differentiation among populations because of its selfing attribute and the barriers of sea and mountain range for gene flow. In addition, the founder effect may be the other reason for this pattern of population genetic structure.

Keywords: green foxtail; *Setaria viridis*; weediness; genetic diversity; population genetic structure; gene flow

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1. Introduction

Setaria viridis (L.) Beauv., or green foxtail, belongs to the grass family Poaceae, and is native to Eurasia [1–3]. It is the putative ancestor of foxtail millet, *Setaria italica* (L.) Beauv., which was domesticated and selected by farmers in northern China about 10,500 years ago, based on archeological evidence [4–7]. Currently, foxtail millet is a minor crop cultivated in India, China and Taiwan for food, and in Europe for birdseed mainly [8]. The world production of millet was around 31 million tons in 2018, and foxtail millet is the largest crops among millets [9]. The cytogenetic study of GISH [10] and the phylogenetic relationship analyzed with chloroplast and nuclear genes [11,12] showed that the genome difference between *S. italica* and *S. viridis* was slightly distinguishable, which is concordant with the fact that few different morphological traits can be observed between them [5,13,14]. Furthermore, there was about 22% of SNP variation detected in both *S. viridis* and *S. italica* in the genome-wide data [15].

Due to its small stature, short lifecycle (6–8 weeks), small genome size (~510 Mb), diploidy ($2n$ = 18), self-pollination, efficient C4 photosynthesis and transformability,

S. viridis has been recommended as a model species for the research of C4 plants, which also include switchgrass, maize and sorghum [1,16–19]. In addition, a new genomic resource of *S. viridis* was released by de novo assembly recently [15]. On the other hand, the whole-genome sequencing of foxtail millet has been released [16,20], and it makes *S. italica* important in model systems as well [17]. Recombinant inbred lines (RILs) of *S. italica* x *S. viridis*, accession A10, have been thoroughly investigated and applied in research on bioenergy, feed and forage of stock [16,17,19,21,22]. Moreover, A10 accession has been established and applied to QTL mapping and the construction of a genetic map [16,22].

The problem of invasive weeds caused losses of agriculture, overdose application of pesticide and more pollution to environments and eco-systems. *S. viridis* possesses the potential to adapt to various environments and habitats; consequently, it has invaded not only throughout the temperate, but in some subtropical and tropical regions of agricultural systems worldwide [8,23–26]. The variable biodiversity in phenotype and genotype allows *S. viridis* to invade, endure and colonize in different local environments [3]. The seeds can float with water flow for 10 days [27] or attach to animals or human clothing with the bristles on the panicles to disperse for a long distance [28–31]. Hence, it is considered as a serious weed for agriculture in many countries, such as Canada, the USA, Spain and Japan [4,30,32,33], and usually grows near riverbanks and lakes, and in roadsides, grain fields, wastelands and any other disturbed regions [34,35]. The invasiveness of *S. viridis* was detrimental to crop growth and production, with one reference of reported losses of 21% and 44% yields of wheat [36]. Furthermore, many other crops, like grain sorghum, rice and alfalfa, were also reported to be influenced by *S. viridis* significantly [28,29,37]. Despite being a noxious weed, *S. viridis* is extensively used as forage in Europe and as herbal medicine in some regions of Asia [38,39]. In North America, *S. viridis* is widely dispersed [30], but it does not tend to occupy high mountains and regions of lower latitudes [40]. Similarly, Jia et al. (2013) found that *S. viridis* was localized to northern latitude regions in China, suggesting that the local populations are more adapted to temperate climates [41].

Due to the interfile between *S. italica* and *S. viridis*, the occurrence of gene flow might make the weediness of *S. viridis* more powerful in nature [14,42]. For example, the giant green foxtail, *S. viridis* var. "major" (Gaud.) Posp., an intermediate type, is originally from the hybridization between wild *S. viridis* and cultivated *S. italica* [14,34,43]. In addition, *S. faberi* R. A. W. Herrma and *S. verticillata* (L.) P. Beauv., the tetraploid species (AABB genome), originated from a natural crossing between *S. adhaerans* (Forssk.) Chiov. and *S. viridis* [10]. On the contrary, it was also reported that reproductive barriers partially existed, resulting in a low level of introgression and gene flow in a wild-weed-crop complex [14,44–46]. It seems that the risk of gene flow between *S. italica* and *S. viridis* is low [47].

Until now, there were some studies about the genetics, genomics and evolution of *S. italica* published, but few about *S. viridis*. Related studies of *S. viridis* did not attract much attention, although it has been recommended as a model system for the studies of C4 photosynthesis, biofuels, and drought and salinity tolerance. The abundance of foxtail millet landraces has been observed in China and Taiwan [48–50]. The intrapopulation genetic diversity of weedy *Setaria* species (*S. viridis*, *S. faberi*, *S. parviflora* (Poir.) Kerguélen, *S. pumila* (Poir.) Roem. and Schult., *S. verticillata*) was quite low, but quite high in inter-population genetic diversity [40,51,52]. Interestingly, the low level of genetic diversity of *S. viridis* was observed in comparison with the other crop wild relatives, such as *Oryza rufipogon* and teosinte [26,53,54]. Instead, the cultivated *S. italica* displayed high diversity compared to sorghum and rice [55,56]. Overall, the genetic diversity and phylogenetic relationship was mostly assessed in the studies of foxtail millet [57–60], and only a few studies also included *S. viridis* collections [12,41,61]. In our opinion, these studies are not enough to deeply understand the genetic diversity and population structure of *S. viridis*, and many questions still remain unanswered. Importantly, that kind of information is crucial for germplasm conservation, genetic mapping, association studies and breeding programs [62,63].

The earliest described specimen of *S. viridis* in Taiwan is the one numbered 107,274 in the herbarium HAST, which was collected in 1916, and the earliest documentation in Taiwan was recorded in 1930 [64]. On the other hand, according to the archeological evidence, the cultivation of foxtail millet in Taiwan could be dated back to 5000 years ago [65]. Foxtail millet is one of the traditional foods for Taiwanese aborigines, and it also was used to brew alcoholic beverages because of the waxy property of Taiwanese landraces [48]. However, genomic or genetic diversity of *S. viridis* and its mechanism of success in becoming an aggressive weed in Taiwan are little understood so far. In this study, we hope to address the following issues: (i) how the pattern of the genetic diversity of *S. viridis* in Taiwan came about; (ii) the estimated population structure of genetic variation and genetic relationship of different *S. viridis* populations in Taiwan; and (iii) the pattern of genetic differentiation among *S. viridis* populations in different geographical regions of Taiwan.

2. Materials and Methods

2.1. Field Collections of S. viridis in Taiwan

The field survey covered Eastern and Western Taiwan, and several offshore islands belonging to the Taiwan Government as well. A total of 141 individuals of *S. viridis* were collected from 10 sites, including four on main island of Taiwan and six distributed on different offshore islands (Table 1). The individuals from the same collection site were regarded as a population. The sample size of every population ranged from 8 to 24. Fresh leaves were dried out using desiccant beads, then preserved at 4 °C. The longitudes and latitudes of all sites were recorded in WGS84 format.

Table 1. Information, including code, site, latitude, longitude and sample size of 10 collection sites in different parts of Taiwan.

Code	Site (Township/County)	Latitude	Longitude	Sample Size
#01	Tongluo, Miaoli	24°29′48.00″ N	120°47′17.00″ E	16
#02	Haiduan, Taitung	23°4′9.00″ N	121°9′33.00″ E	8
#03	Taitung, Taitung	22°43′36.00″ N	121°5′48.00″ E	16
#04	Ludao, Taitung	22°40′25.00″ N	121°28′17.00″ E	9
#05	Liujiao, Chiayi	23°30′56.00″ N	120°17′56.00″ E	10
#06	Qimei, Penghu	23°12′18.00″ N	119°25′37.00″ E	15
#07	Huxi, Penghu	23°35′40.00″ N	119°36′47.00″ E	11
#08	Jinning, Kinmen	24°27′20.00″ N	118°19′8.00″ E	16
#09	Jinsha, Kinmen	24°29′36.00″ N	118°24′44.00″ E	24
#10	Nangan, Lienchiang	26°8′45.00″ N	119°54′47.00″ E	16
Total				141

2.2. Genotyping by Microsatellite

The dried leaf of each sample was homogenized using tungsten carbide beads with Tissuelyser, then the genomic DNA was extracted by using the TPS method with modifications [66]. Twenty-five markers were used at first based on the PIC (polymorphism information content) value reported by Zhang et al. [67].

The forward and reversed primers were elongated with 5′ ACGACGTTGTAAAA 3′ and reversed 5′ CATTAAGTTCCCATTA 3′ sequences, respectively, to perform multiplex-ready PCR [68]. The PCR reaction mixture of each sample was 10 µL in total volume, containing 20 ng template DNA, 1 µL 1× IMMOLASE buffer (BIOLINE), 0.2 mM dNTP, 2.0 mM $MgCl_2$, 40 nM primers, 80 nM fluorophores (VIC, 6-FAM, NED, or PET), 0.05 µL IMMOLASE DNA polymerase (BIOLINE) and 4.55 µL ddH_2O. The amplification program was performed with the following steps: 95 °C 10 min, 20 cycles of 92 °C for 30 s, 63 °C for 90 s, 72 °C for 60 s, then 40 cycles of 92 °C for 15 s, 54 °C for 30 s and 72 °C for 60 s. The four PCR products were pooled together at a ratio of 2:3:4:6 (VIC:6-FAM:NED:PET) in volume, then we used GSLIZ600 as internal control. The size of the SSR fragments was detected by capillary electrophoresis. An elite variety of foxtail millet (*Setaria italica*) in

Taiwan, Taitung Number 8 (TT8), was also pooled into each plate to correct the size bias among different plates. The FSA (.fsa) files that contained fragment sizes and fluorescence intensities were analyzed using the "Fragman" v1.0.9 package in R software v4.0.2 [69,70]. After filtering out the markers with multiple peaks (multiallele), a set of 13 SSR markers distributed on nine chromosomes was selected and used for further analysis.

2.3. Genetic Diversity and Clustering Analysis

The genetic diversity of each marker and sampling site were evaluated with the parameters of allele numbers (Na), effective allele numbers (Ne), observed heterozygosity (Ho) and expected heterozygosity (He), which is also called gene diversity [71], using the "PopGenReport" v3.0.4 package in R software [72]. Additionally, the polymorphism information content (PIC) of each marker was calculated with the "polysat" v1.7.4 package in R software [73].

As for the clustering analysis of 141 *S. viridis* samples, the genetic distance between each pair of individuals was estimated by Euclidean distance using the "adegenet" v2.0.1 package in R software [74]. Subsequently, the distance matrix was used for a principal coordinate analysis (PCoA) using the "cmdscale" function and for constructing a neighbor-joining tree with the "ape" v5.4 package in R software [75]. The variety TT8 was included as an outgroup for phylogenetic tree.

The term "population structure" was defined as the genetic background of all 141 *S. viridis* individuals and estimated with STRUCTURE v2.3.4 using an admixture model [76]. The term "subpopulation" was defined as the clusters based on the optimum clustering number (*K* value). The *K* value was tested from 1 to 11 to find the best *K* for 141 *S. viridis* individuals. For each *K* value, burn-in period and Markov chain Monte Carlo (MCMC) were both set to 100,000× with 20 replications. The result of STRUCTURE was integrated with the "pophelper" v2.3.0 package in R software [77], and the optimum *K* value was determined by the value of delta *K* described in the Evanno method [78]. Finally, the results above were visualized using the "ggplot2" [79] and "ggtree" v3.11 packages in R software [80]. The individuals in the barplot of STRUCTURE were sorted by 10 collection sites, from #01 to #10. The different genetic background of each collection site based on structure analysis were represented by pie charts on the map of Taiwan.

2.4. F-Statistics of Populations at 10 Collection Sites

Wright's *F*-statistics, including F_{IS}, F_{ST} and F_{IT}, were used to summarize the population structure among the 10 collection sites [81]. The estimator of each parameters was described by Weir and Cockerham [82]. The F_{ST}-derived estimator, Nm value [83], was further used to evaluate the gene flow between each pair of collection sites under the island model theory. The formula used was $Nm = (1 - F_{ST})/4F_{ST}$ [81]. Finally, all of the *F*-statistics parameters were calculated in R software.

3. Results

3.1. Molecular Diversity of Microsatellite Markers from the Individuals of Green Foxtail in Taiwan

Thirteen SSR markers were used to assess the genetic diversity of 141 *S. viridis* individuals in Taiwan (Table 1). A total of 79 alleles were detected, with an average of 6.1 alleles per locus. The observed allele number ranged from 2 (SICAAS5005, SICAAS6052, SICAAS7090, SICAAS9121) to 16 (SICAAS1065). On the other hand, the effective allele number (Ne) was predicted when the number of alleles with equal frequency was assumed. In average, 3.558 effective alleles per locus were measured in all *S. viridis* populations. The marker SICAAS1015 showed the largest Ne (9.18), and SICAAS5005 showed the smallest Ne (1.26). The value of observed heterozygosity (Ho) ranged from 0.015 to 0.022 for all markers. Compared to the expected heterozygosity (He), the mean of Ho was only 0.0054, indicating the characteristic of high self-pollinated rate of *S. viridis*. In addition, nine out of 13 SSR markers displayed none of the observed heterozygosity in our study. Conversely, the values of expected heterozygosity (He) ranged from 0.21 (SICAAS5005) to 0.89

(SICAAS1015). Four markers, SICAAS1015, SICAAS1065, SICAAS2084 and SICAAS3090, exhibited more abundant gene diversity in our study. The PIC values ranged from 0.18 (SICAAS5005) to 0.88 (SICAAS1015) with a mean of 0.527, indicating that highly polymorphic SSR markers were used in our study. The marker SICAAS5005 showed the lowest PIC value, and provided little information for the analysis. The PIC values of seven markers were within the range of 0.25 to 0.5, displaying moderate polymorphic information. Finally, the PIC values of five markers were over 0.7, representing high polymorphism in our study (Table 2).

Table 2. Genetic diversity parameters of 13 SSR markers used to assess 141 *Setaria viridis* individuals.

Markers	Na	Ne	Ho	He	PIC
SICAAS1015	14	9.18	0.000	0.89	0.88
SICAAS1065	16	7.79	0.015	0.87	0.86
SICAAS2084	8	5.16	0.000	0.81	0.78
SICAAS3090	10	6.06	0.022	0.83	0.81
SICAAS5005	2	1.26	0.000	0.21	0.18
SICAAS5081	4	2.19	0.000	0.54	0.48
SICAAS6052	2	1.37	0.000	0.27	0.30
SICAAS7002	7	4.35	0.017	0.77	0.73
SICAAS7008	4	1.98	0.000	0.49	0.44
SICAAS7090	2	1.41	0.000	0.29	0.27
SICAAS8025	4	2.12	0.016	0.53	0.41
SICAAS9121	2	1.41	0.000	0.29	0.26
SICAAS9130	4	1.98	0.000	0.50	0.45
Average	6.1	3.558	0.0054	0.561	0.527

Na: observed allele number; Ne: effective allele number; Ho: observed heterozygosity; He: expected heterozygosity; PIC: polymorphic information content.

The genetic diversity indexes of *S. viridis* populations collected from 10 sites were revealed in this study (Table 3). Among all 10 collection sites, the number of observed alleles ranged from 13 (sites #01 and #02) to 29 (site #08), with a mean of 18.9. Similarly, the largest number of effective alleles was also detected at site #08, and the lowest was at both site #01 and #02. Overall, 15.619 effective alleles per site were found. The observed heterozygosity (Ho) was only detected at three sites (#03, #08 and #10) with very small values, and the other populations of *S. viridis* showed none of Ho. The mean of Ho was 0.005, which was expected due to the characteristics of self-pollination of *S. viridis*. As for the expected heterozygosity (He), it ranged from 0.03 (site #04) to 0.27 (site #08), and no He was observed at two collecting sites (#01 and #02).

Table 3. Genetic diversity index of *Setaria viridis* individuals in each collecting site.

Collecting Site	SS	Na	Ne	Ho	He	F_{IS}	F_{ST}	F_{IT}	Nm
#01	16	13	13.00	0.00	0.00	0.9896	0.4895	0.9947	0.26
#02	8	13	13.00	0.00	0.00	0.9901	0.4995	0.9950	0.25
#03	16	19	14.25	0.01	0.12	0.9841	0.3954	0.9904	0.38
#04	9	14	13.60	0.00	0.03	0.9904	0.4783	0.9950	0.27
#05	10	22	18.63	0.00	0.16	0.9921	0.3540	0.9949	0.46
#06	15	22	14.94	0.00	0.11	0.9905	0.4476	0.9948	0.31
#07	11	20	15.57	0.00	0.12	0.9915	0.3984	0.9949	0.38
#08	16	29	21.82	0.02	0.27	0.9723	0.2525	0.9793	0.74
#09	24	18	15.04	0.00	0.07	0.9901	0.4277	0.9943	0.33
#10	16	19	16.34	0.02	0.14	0.9653	0.3943	0.9790	0.38
Average		18.9	15.619	0.005	0.102				

SS: sample size; Na: observed allele number; Ne: effective allele number; Ho: observed heterozygosity; He: expected heterozygosity; F_{IS}: inbreeding coefficient of an individual relative to the subpopulation; F_{ST}: the degree of genetic differentiation between populations; F_{IT}": inbreeding coefficient of an individual relative to the total population; Nm: number of immigrants, the extent of gene flow.

3.2. Genetic Relationship of Green Foxtail Individuals Collected in Taiwan

The results of the principle coordinate analysis offer preliminary insight on the genetic relationship among 141 *S. viridis* individuals or 10 wild populations collected in Taiwan (Figure 1). The first and the second coordinates explained 23.00% and 14.90% of variability, respectively. First of all, sites #01 and #05, which are both in Western Taiwan, were grouped together. Secondly, three collecting sites, all in Taitung County (sites #02 and #03), including small island Ludao (site #4), had a slightly close relationship with each other. However, two individuals at site #03 were slightly away from the others. Interestingly, the *S. viridis* at site #06 and site #07 (Qimei and Huxi in Penghu, respectively) were significantly isolated from the other sites. However, one collection belonging to site #06 was closer to the individuals at sites #01 and #05. Finally, three outer collection sites (#08, #09 and #10) closer to mainland China showed an overlapped pattern with each other that indicated a closer relationship among them.

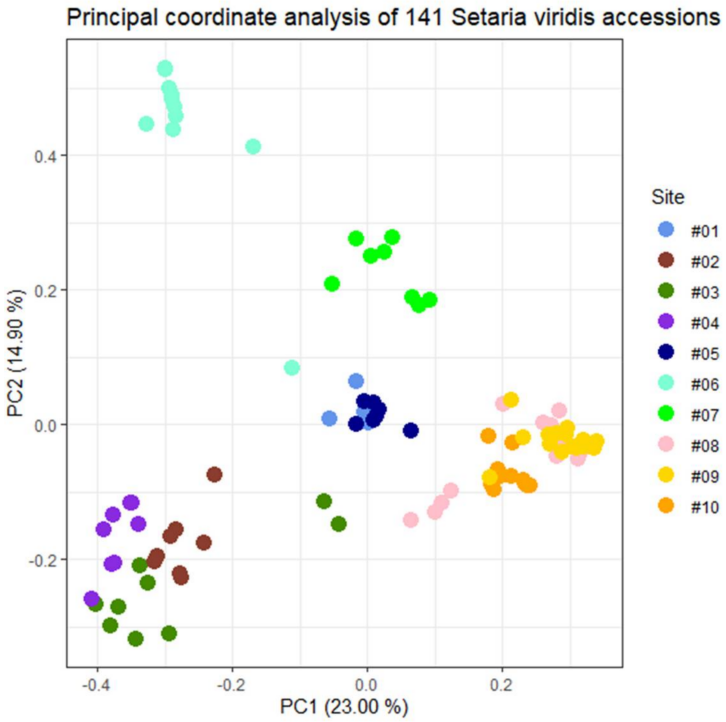


Figure 1. Principal coordinate analysis of the 141 *Setaria viridis* individuals in Taiwan. The first and the second coordinates explained 23.00 % and 14.90 % of variability, respectively. The 10 collecting sites are marked by different colors.

A similar pattern with more details is presented in the phylogenetic tree (Figure 2). Individuals collected in Taitung County, including sites #02, #03 and #04, were in Clade I. Broadly, *S. viridis* individuals collected at site #04 (Ludao) were closer to collections at site #03 (Taitung), however, several individuals collected at site #03 showed a complex and mixed relationship among these populations. Individuals from site #01 (Miaoli) and #05 (Chiayi), with clear geographical separation, were grouped in Clade II. Nevertheless, an individual from site #06 (Penghu) was grouped in this clade, indicating the probable movement behavior of *S. viridis* individuals could occur between Penghu and Chiayi occasionally. Besides, some individuals from site #08 were closer to site #09 (see Clade III), and the others were closer to site #10 instead (see Clade IV), suggesting the higher abundance of *S. viridis* at site #08 (Jinning, Kinmen). Furthermore, the individuals from site #09 (Jinsha, Kinmen) showed clear separation from the individuals from site #10 (Lienchang). Moreover, the individuals from site #09 can be further divided into two small clusters. The individuals from site #07 (Huxi, Penghu) were also isolated as Clade V. Similarly, most

S. viridis individuals from site #06 were obviously separated from the others (as Clade VI), but an exception was grouped in Clade II and closer to the individuals at site #05. This is consistent with the result of the PCoA (Figure 1).

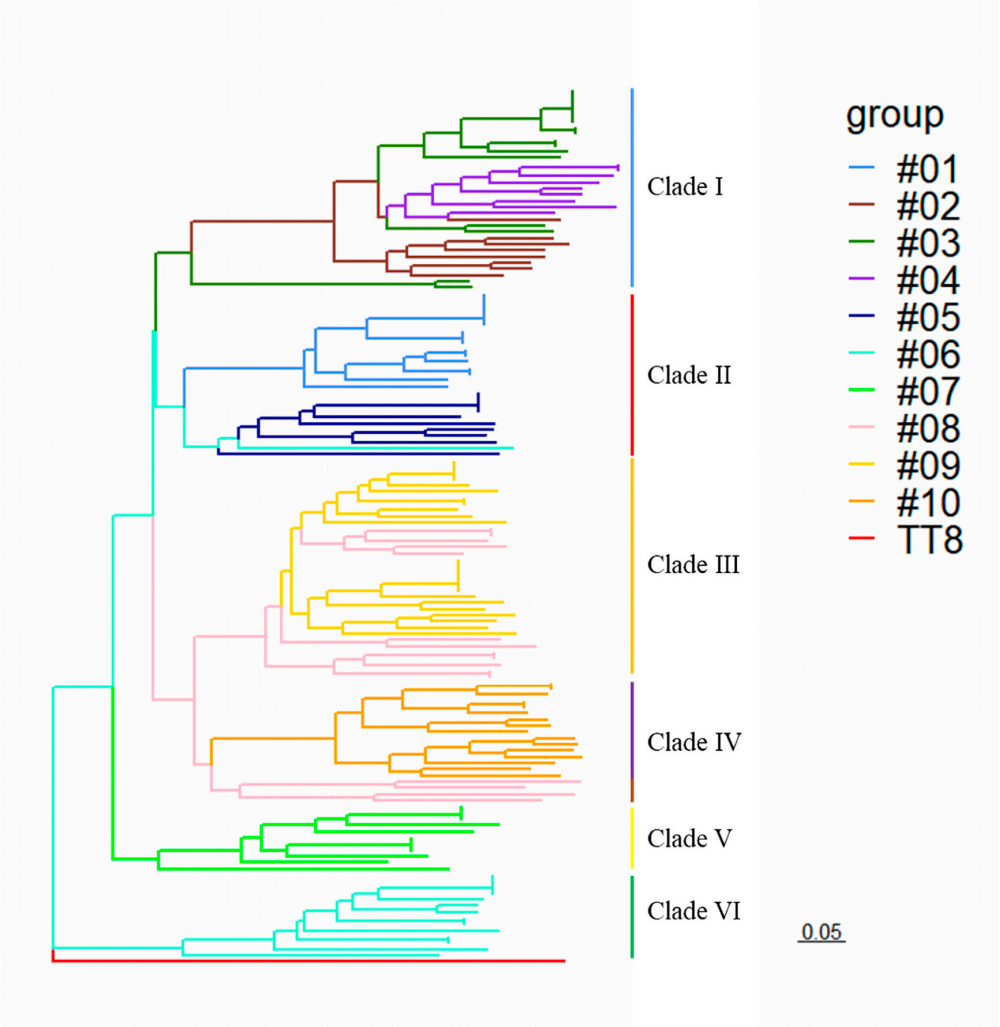
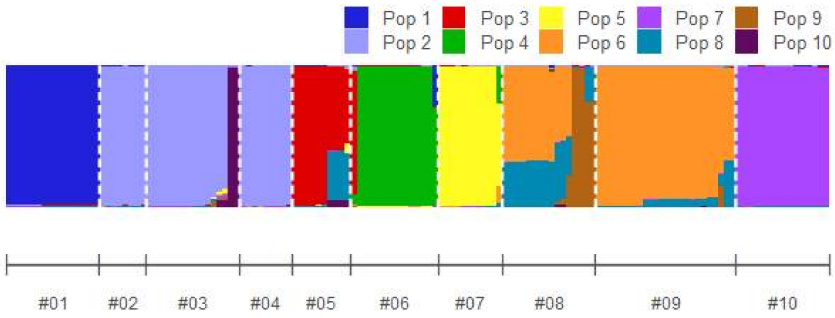


Figure 2. Neighbor-joining tree of the 141 *Setaria viridis* accessions in Taiwan. The color of each clade represents each individual collected from its own collecting site. The vertical line with different colors of every clade number corresponds to subpopulations that were inferred by the STRUCTURE analysis.

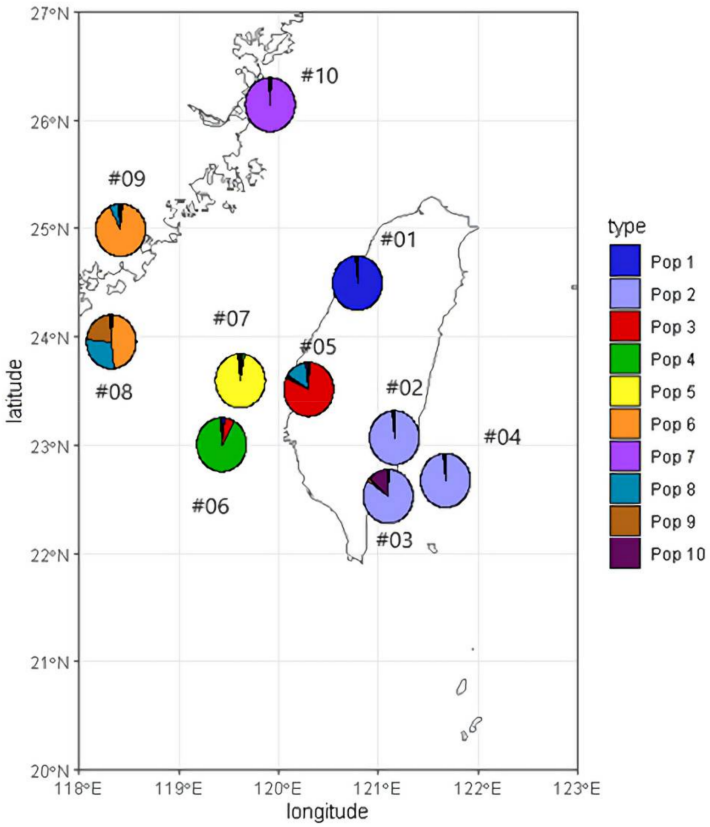
3.3. Population Structure and Geographic Distribution

The population structure of *S. viridis* collected from 10 sites was simulated by STRUCTURE (Figure 3a). The *K* value was inferred by the Evanno method, from $K = 1$ to $K = 11$ (Figure S1). The best *K* value was 10; however, 10 colors of different genetic backgrounds did not exactly represent the 10 collection sites (Figure 3b). In addition, the admixed individual is defined when the percentage of its own genetic background does not exceed at least 80%. The wild populations collected at sites #01, #02, #04 and #10 possessed pure genetic backgrounds with no admixture. In contrast, four individuals from site #05, three from #09, two from #06, one from #07 and most individuals from #08 were considered as admixture. Above all, the individuals collected from site #08 (Jinning, Kinmen) displayed the most complicated pattern (Figure 3b). The individuals from site #01 (Miaoli) and site #10 (Lienchiang) independently formed distinct subpopulations, called Pop 1 and Pop 7, respectively. Moreover, the individuals from sites #02, #03 and #04, in Eastern Taiwan (Taitung) were all grouped in Pop 2. Interestingly, two individuals from site #03 excep-

tionally formed another subgroup (Pop 10). The individuals from site #05 (Chiayi) were all grouped in Pop 3, mixed with another genetic background that also appeared in Pop 8 (in greenish blue), indicating potential gene flow or introgression. Surprisingly, two collecting sites of *S. viridis* from Penghu (sites #06 and #07) clearly formed two different subpopulations, Pop 4 for site #06 and Pop 5 for site #07 (see Figure 3a and Table 1). In addition, one individual collected at site #06 was grouped in Pop 3 instead of Pop 4, and showed high percentage of red background, which can be also noticed in the phylogenetic tree (Figure 2) and the PCoA (Figure 3). Finally, the individuals from sites #08 and #09 (Kinmen) were grouped in Pop 6, but displayed the mixed genetic background of Pop 8 and Pop 9.



(**a**)



(**b**)

Figure 3. (**a**) STRUCTURE barplot of the 141 *Setaria viridis* individuals with *K* value = 10 inferred by the Evanno method. The order of results from STRUCTURE is sorted by 10 collection sites from #01 to #10. An individual is considered as the admixture when the genetic background is lower than 80% of its own percentage. (**b**) Geographic distribution of the 10 collection sites in Taiwan. The pie chart at each site represents the genetic proportion inferred by STRUCTURE when *K* = 10.

3.4. Genetic Differentiation among the Populations of S. viridis in Taiwan

F-statistics were also estimated in this study. All F_{IS} and F_{IT} values for the 10 sampling sites were all over 0.96. The lowest value of F_{IS} (0.9653) and F_{IT} (0.9790) were observed at site #10 (Lienchiang), an offshore island of Taiwan that is close to mainland China. The highest F_{IS} (0.9921) was detected at site #05 (Chiaiyi), and the highest F_{IT} (0.9950) was detected at sites #02 and #04 (Taitung). Overall, it was concluded that the wild *S. viridis* in Taiwan are highly self-pollinated within a population, which agreed with the result of low observed heterozygosity (Table 3). The extent of genetic differentiation between populations was estimated by F_{ST} value. We found high genetic differentiation among wild *S. viridis* populations in Taiwan. The populations at site #02 (Taitung) and site #01 (Miaoli) displayed the highest F_{ST} values, 0.4995 and 0.4895, respectively. Oppositely, site #08 (Kinmen) displayed a relatively smaller F_{ST} value (0.2525), suggesting that more gene flow events might take place in this area. Likewise, the number of immigrants (Nm) denotes the extent of gene flow, in which great differences of Nm value were found (0.25 to 0.74). Obviously, the highest extent of gene flow was detected at site #08, Kinmen (0.74), and smaller Nm values were observed at sites #01, #02 and #04, Taitung (0.25 to 0.27), which is in accordance with the results of genetic differentiation (Table 3).

3.5. Pairwise F_{ST} Index of Collecting Populations in Taiwan

Pairwise F_{ST} value, representing the extent of genetic differentiation between populations, was assessed for the 10 collection sites, and ranged from 0.14 to 0.62 (Table 4). The least extent of genetic differentiation was between sites #08 and #09 (Kinmen), indicating gene flow events might happen more frequently here. Next, the *S. viridis* populations collected at sites #03 (Taitung) and #04 (Ludao) also showed a low level of genetic differentiation (F_{ST} = 0.17). The F_{ST} values among sites #02, #03 and #04, in Taitung, were quite small in comparison with the others. On the contrary, the highest level of genetic differentiation was observed between site #07 (Huxi, Penghu) and site #04 (Ludao, Taitung), which are both small offshore islands off Taiwan's main island (Figure 3b). Next, three combinations from different collection sites showed the second-highest genetic differentiation (F_{ST} = 0.59): #07 (Penghu) and #02 (Taitung), #09 (Kinmen) and #06 (Penghu), and #10 (Lienchiang) and #06 (Penghu). In addition, two collection sites, #06 and #07 (Penghu), showed relatively high genetic differentiation with the other populations. On the contrary, the *S. viridis* population at site #08 (Jinning, Kinmen) exhibited the opposite result in our investigation. For example, the populations collected at sites #05 (Chiayi), #10 (Lienchiang) and #07 (Huxi, Penghu) separately showed the least genetic differentiation with site #08, suggesting that the composition of *S. viridis* at site #08 is more complex than the others. Finally, the population collected at site #01 (Miaoli) also showed the least genetic differentiation (F_{ST} = 0.36) with the population at site #05 (Chiayi), which also is located in Western Taiwan (Figure 3b).

Table 4. Pairwise F_{ST} value between subpopulations from the 10 collecting sites.

	#01	**#02**	**#03**	**#04**	**#05**	**#06**	**#07**	**#08**	**#09**
#02	0.47								
#03	0.54	0.22							
#04	0.55	0.28	0.17						
#05	0.36	0.47	0.46	0.51					
#06	0.57	0.52	0.56	0.52	0.50				
#07	0.58	0.59	0.58	0.62	0.50	0.50			
#08	0.41	0.38	0.42	0.44	0.32	0.49	0.40		
#09	0.52	0.49	0.55	0.55	0.42	0.59	0.50	0.14	
#10	0.51	0.52	0.55	0.58	0.46	0.59	0.54	0.32	0.45

4. Discussion

4.1. Molecular Diversity of Microsatellite from the Individuals of Green Foxtail in Taiwan

Simple sequence repeats (SSRs) are very useful for the investigation of population genetics and studies of evolution because they is easy to manipulate and contain abundant molecular variations and information [84–86]. In this study, 6.1 allele numbers were observed from 141 *S. viridis* individuals with 13 SSR markers. Despite the estimated PIC value, 0.527 on average, being lower than that in other related studies of *S. viridis* or *S. italica* [44,55,57], highly polymorphic markers were still used in our analysis (Table 2). The other related grass species of *S. viridis*, such as *Miscanthus sinensis* and switchgrass, showed different patterns. The naturalized populations of *M. sinensis* in the United States contained only 2.3 alleles per locus by 74 molecular markers, and the PIC value ranged from 0.2228 to 0.3030 [87]. Nevertheless, 8.7 alleles per locus were detected from 156 switchgrass individuals with 18 highly polymorphic markers [88]. Therefore, we thought that the quality of used markers was much more important than the quantity of markers. In our analysis, we preferred to select highly informative markers because it was reported that a small number of molecular markers with high quality can be enough for analysis of genetic variation [89]. Only four markers (SICAAS1065, SICAAS3090, SICAAS7002, SICAAS8025) displayed low extent of the observed heterozygosity (Ho), indicating that in Taiwan, *S. viridis* is a highly selfing species, which corresponds with previous studies [26,40]. In addition, a similar pattern of Ho value in terms of *S. viridis* at different collection sites was also observed (Table 3). On the other hand, importantly, the advantages of low heterozygosity (high rate of selfing) includes the ease of sequencing, and better analyzing and understanding of genetic and evolutionary mechanisms [90,91]. Besides, an effective allele number (Ne) based on the frequency of variants is an alternative measure of intrapopulation diversity [92]. Hence, it basically corresponds to the expected heterozygosity (Table 2). An average effective allele number of 3.558 can be seen in our *S. viridis* populations in terms of the assessment of 13 SSR markers. Interestingly, the Ne value of *S. viridis* in Taiwan was higher than that in mid/southern US, but much lower than the Asian populations [89].

For the purpose of better understanding the relationship between the genetic diversity and geographical distribution, we mainly focused on the genetic diversity of *S. viridis* populations from different parts of Taiwan (Table 3). The highest value of observed allele number (Na), effective allele numbers (Ne) and expected heterozygosity of *S. viridis* population were all detected at site #8 (Kinmen), indicating the highest genetic diversity of *S. viridis* was preserved in a population on Kinmen Island, near mainland China, which might be one of the origin of *S. viridis* (cp. [1,3]). On the contrary, the lowest genetic diversity was observed at site #1 (Miaoli) and site #2 (Taitung) in terms of the relatively low Na, Ne and He. The relatively low genetic diversity of intrapopulation of *S. viridis* was reported previously [40,51,52]. Wild sorghum populations collected from different regions in Africa showed high genetic diversity, He = 0.46 on average, assessed by six SSR markers [93]. Likewise, the *M. sinensis* population collected in the US showed a medium level of genetic diversity, with He values ranging from 0.2776 to 0.3738 [87]. However, in this study of *S. viridis* in Taiwan, the level of heterozygosity was considerably low in comparison with previous studies (cf. [41,89]).

4.2. Genetic Relationship and Structure of Green Foxtail Individuals among Different Parts of Taiwan

Basically, both the PCoA and phylogenetic analysis showed a similar pattern of results, which is accordance with geographical distribution and concordant with previous studies. The main island of Taiwan extends 394 km along its north–south oriented axis and a width of 140 km at its broadest. There are 268 peaks above 3000 m in elevation, and all of them are located within the so-called Central Range, which basically follows the axis of the island. No doubt this Central Range is a barrier for the gene flow between the east and west parts of Taiwan [94]. In addition to the isolation by the Taiwan Strait, the Central Range on the main island of Taiwan is also a barrier for gene flow. The *S. viridis* were also

found to be grouped by regions or species in phylogenetic tree in a previous study [61]. Moreover, our results were concordant with the PCA pattern from the world-wide *S. viridis* panel in terms of SNP, PAV and structural variant genetic structure (cf. [15]). As a result, the genetic relationship may be influenced by geographical distribution for the same species [95]. In this study, some individuals from different populations clustered together in the same clade, displaying the mixed genetic branches of *S. viridis* collected at different sites in the phylogenetic tree (Figure 2). This might be the result of migration caused by human activities and long-distance animal movements. For example, there is routine transportation by ferry between Taitung City (site #03) and Ludao (site #04). The same ferry shuttle also ran between Chiayi (site #05) and Huxi (site #07). The simulated genetic backgrounds basically agreed with the administrative districts of Taiwan, except that Pop 4 and Pop 5 were two separate townships in Penghu (Figure 3a). Notably, four out of 10 *S. viridis* samples in Chiayi (site #05) showed admixed genetic background, which was also found at sites #08 and #09 in Kinmen. In addition, relatively low genetic differentiation between sites #05 and #08 was detected (Table 4), implying that some of the *S. viridis* in Chiayi might be taken from Kinmen because of human activities. Finally, pure and different genetic backgrounds at collection sites suggested that gene flow rarely happened among them, causing the pairwise genetic differentiation to be quite high. It was concluded that the *S. viridis* at these sites mainly reproduced by selfing, and it was hard for them to disperse by pollens or seeds of their own. Finally, the *S. viridis* individuals in the population at site #08 (Jinning, Kinmen) mixed in clade III and IV, showing the most complex genetic variation in our study in terms of the analysis of genetic-diversity parameters, population structure and genetic relationship (Table 3, Figure 3a).

4.3. Genetic Differentiation and Possible Gene Flow among the Populations of Different Collection Sites

F-statistics is one of the major measures to examine the differentiation between a subdivided population that deviates from the Hardy–Weinberg equilibrium. Briefly, F_{IS}, F_{IT} and F_{ST} represent the genetic diversity of different types of conditions that are individuals within the subpopulation, individuals within the total population and subpopulation within the total population, respectively [81,82]. In this study, low F_{IS} and F_{IT} values were observed at sites #08 (Kinmen) and #10 (Lienchiang), indicating that the inbreeding coefficient of *S. viridis* collected at sites #08 and #10 were relatively low. Consequently, the observed heterozygosity of *S. viridis* at these two sites were essentially high (Table 3). The extent of genetic differentiation (F_{ST}) among populations varied up to two times. *S. viridis* collected at site #08 showed the lowest genetic differentiation (F_{ST} = 0.2525), and the population at site #05 was ranked second (F_{ST} = 0.3540), indicating that gene flow in Kinmen and Chiayi might have occurred more frequently. Oppositely, the collected populations at site #01 (F_{ST} = 0.4895) and site #02 (F_{ST} = 0.4995) showed high extents of genetic differentiation, which might be due to less chance of interflow with other populations (Table 3). The higher Nm values at sites #05 and #08 were observed; however, the two admixed populations contained elevated heterozygosity (He), indicating gene flow might have taken place at these two sites recently [96,97]. Instead, the *S. viridis* at site #09 showed a relatively higher Nm value (0.33), but lower heterozygosity (0.07), which may be the result of ancient crossing, then followed by inbreeding with no cross-pollination [26]. It was reported that elevated diversity might be the result of multiple origins or larger populations [15]. The collection sites with no admixed individuals (sites #01, #02 and #04), exhibited a lower Nm value (<0.3), suggesting that less gene flow occurred before.

Pairwise F_{ST} varied dramatically, 0.14 to 0.62 (Table 4). The higher extent of genetic differentiation between the *S. viridis* populations was mostly observed at sites #06 or #07 compared with other collection sites. For example, the highest one (F_{ST} = 0.62) was detected between site #07 (Huxi, Penghu) and site #04 (Ludao, Taitung), two offshore islands of Taiwan. Likewise, some studies found that *S. viridis* both in Eurasia and North America displayed strong intracontinental differentiation [40,95]. In addition, the populations at site #09 (Kinmen) and #10 (Lienchiang) also showed the same extent of high genetic

differentiation (F_{ST} = 0.59) as site #06. As a result, the *S. viridis* populations collected at sites #06 and #07 seemed to be more departed from the other populations in Taiwan, which agreed with the patterns of the PCoA and the phylogenetic tree (Figures 1 and 2). In fact, site #06 (Qimei) and site #07 (Huxi) belong to the Penghu archipelago, which is located in the middle of the Taiwan Strait (Table 1). One possible reason could be that the special climate of Penghu could have selected or filtered certain genotypes or genes, which is the same condition in North America (cf. [15,89]). Oppositely, the smallest F_{ST} was observed between sites #08 and #09 (F_{ST} = 0.14), followed by the ones between sites #03 and #04 (F_{ST} = 0.17), which are located in the same counties, Kinmen and Taitung, respectively. On the other hand, the collection sites in the same administrative district, such as sites #02, #03 and #04 (Taitung), and sites #08 and #09 (Kinmen), revealed less genetic differentiation than the others, indicating that isolation by distance or geographic barriers may affect the possibility of migration or exchange [98], such as the spread of seeds or pollens by wind and animals in nature. The other explanation might be that more frequent human activities take place within the neighboring area, which then increase the probability of artificial and unintentional movement of caryopses and individuals [99,100]. For example, there are routine ferry shuttles between the regions of sites #03 and #04.

Sites #05 and site #08 displayed relatively less genetic differentiation compared to other sites, which is congruent with their higher values of Nm (Table 3). Interestingly, the genetic distance between *S. viridis* and *S. italica* in the same area was lower than that between different areas of the same species, indicating the geographical factors was more important in shaping genetic differentiation than taxonomical factor between *S. viridis* and *S. italica* [95]. The F_{ST} value between *S. viridis* and *S. italica* (at the taxonomical level) was 0.05 to 0.41 in a previous study, which was lower than that of our study (at the geographical level) [56]. On the contrary, different races or species of rice showed significantly genetic differentiation (F_{ST} = 0.1166 to 0.42). The F_{ST} value of *Sorghum halepense* populations collected in the US were quite low, indicating not much differentiation among wild sorghum populations from different states in the US [101].

In conclusion, *S. viridis* showed a pattern of low diversity and heterozygosity within a population, but high differentiation among populations. This is accordant with a similar previous study of a Taiwanese coastal herbal plant, *Lysimachia mauritiana*, with allozyme markers [102]. In *L. mauritiana*, allozyme variation was very low, and *F*-statistics indicated an extremely high level of population differentiation, implying limited gene flow among populations. This pattern of population genetic structure probably resulted from severe genetic drift triggered by genetic bottlenecks, suggesting that Taiwanese populations were likely to be derived from four independent founder events [102]. Several selective forces could influence the population structure, genetic diversity and differentiation of *Setaria* grass were mentioned in [3]. For example, the decreased allele richness of *S. viridis* samples in North America was the result of the founder effect or genetic drift. A strong population structure of *S. viridis* had been presented in the US and Canadian collections because of different climatic zones or latitude [26,89,95]. Instead, the populations collected from China lacked an obvious population structure [41,89,103]. Surprisingly, the *S. viridis* populations in Taiwan, a small island, still displayed a strong population structure (Figure 3a). Up to 84 % of the *S. viridis* samples were considered as pure individual, indicating a high selfing rate and low extent of gene flow occurred among populations in Taiwan. However, most of admixed individuals were found at site #08, probably due to the short distance away from mainland China, resulting in a more complex genetic background than the other populations in Taiwan. The extent of genetic diversity was essentially low, but displayed strong population structure and differentiation [104–106], suggesting that not only normal, but specifically adapted genotypes still existed in the *S. viridis* populations in Taiwan (cp. [3]).

Investigation of the genetic structure, differentiation and related pattern of geographical distribution can help us to better understand the domestication and evolutionary mechanism [107]. Moreover, the *S. viridis* collections with abundant diversity and charac-

teristics could be a valuable resource for breeding programs of foxtail millet, as is planned or is already underway globally for other crop wild relatives (CWR) for the purpose of enhancing genetic resources, including those at risk of extinction [108–110]. Finally, the related knowledge needs to be re-examined at a genome-wide level, and even include more geographic areas with larger collections to investigate subtler population structure and obtain a better diversity panel that can be applied in GWAS (Genome-wide association study) and breeding program in the future.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/d13040159/s1, Figure S1: Delta K value from $K = 2$ to $K = 10$ evaluated by the Evanno method.

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